

PERIVASCULAR MESENCHYMAL PRECURSOR CELLS

FIELD OF THE INVENTION

This invention relates to mesenchymal precursor cells, and the isolation of a subpopulation
5 of such precursors carrying a perivascular marker.

BACKGROUND OF THE INVENTION

Numerous attempts at isolating and enriching mesenchymal precursor cells have been
attempted because of the potential that these cells have for medicinal use. Pittinger *et al.*,
10 (1999) show the expansion of clonogenic cells from bone marrow and describes a
preparation of enlarged mesenchymal stem cells. A more recent example of such a method
providing for a relatively high yield from bone marrow is disclosed in publication
WO01/04268 to Simmons *et al.*,

15 To date however there have been no examples of methods that permit the isolation of
mesenchymal precursor cells from a wide range of tissues.

SUMMARY OF THE INVENTION

The present invention arises from the finding that a population of multipotential mesenchymal
20 precursor cells (MPCs) is present in a perivascular niche. This has led to the demonstration
that there is a much wider range of tissue type sources of MPCs than the single tissue, bone
marrow, referred to in WO01/04268. The present invention arises from the additional
finding that an enriched population MPCs can be differentiated into two populations
discriminated by the marker 3G5. MPCs that are 3G5 positive are considered of interest
25 particularly for neovascularization applications, although demonstrably they are also shown
to differentiate into other tissue types. It is an additional finding of the present invention that
levels of MPCs present in preferred enriched populations of this invention are able to give
rise to sufficient numbers of committed cells to provide a number of differentiated tissue
types.

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In a first form of a first aspect the invention might be said to reside in a method of enriching for mesenchymal precursor cells (MPCs), the method including the step of preparing a single cell suspension from a vascularised source tissue and the step of enriching based on the presence of an early perivascular cell marker.

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In a second form of the first aspect the invention might be said to reside in a method of enriching for mesenchymal precursor cells, the method including the step of preparing a single cell suspension from a, non-bone marrow, vascularised source tissue and separating the tissue into separate cells and the step of enriching based one of the presence or level of one or more early developmental markers and the absence of one or more surface markers indicative of commitment.

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In a third form of the first aspect the invention might be said to reside in a method of enriching for mesenchymal precursor cells (MPCs), the method including the step of preparing a single cell suspension from a vascularised source tissue and the step of enriching based on the presence of markers expressed in the vascularized tissue by peri-vascular cells.

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In a second aspect the invention might be said to reside in an enriched population of cells enriched for mesenchymal precursor cells (MPCs) said MPCs having a phenotype of 3G5, MUC18, VCAM-1, STRO-1^{bri} and α smooth muscle actin.

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In a first form of a third aspect the invention might be said to reside in an isolated mesenchymal precursor cells (MPCs) said MPCs having a phenotype of 3G5, MUC18, VCAM-1, STRO-1^{bri} and α smooth muscle actin.

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In a second form of the third aspect the invention might be said to reside in an isolated mammalian cell that is multipotent and that is positive for the surface marker 3G5.

In a third form of the third aspect the invention might be said to reside in a mesenchymal precursor cell (MPC), capable of forming a clonogenic colony and differentiating to three or more mesenchymal tissue types, isolated from a tissue of the group comprising, but not limited to, adipose tissue, teeth, dental pulp, skin, liver, kidney, heart, retina, brain, hair
 5 follicles, intestine, lung, spleen, lymph node, thymus, pancreas, bone, ligament, bone marrow, tendon, and skeletal muscle, and which is positive for the surface marker STRO-1.

In a fourth form of the third aspect the invention might be said to reside in an unexpanded population of cells enriched for mesenchymal precursor cells (MPCs), capable of forming a
 10 clonogenic colony and differentiating to three or more mesenchymal tissue types, said MPCs co-expressing the surface markers MUC18/CD146 and alpha-smooth muscle actin.

In a fourth aspect the invention might be said to reside in a differentiated progeny cell arising from the third aspect of the invention preferably wherein the progeny cell is at least an
 15 osteoblast, odontoblast, dentin-producing, chondrocyte, tendon, ligament, cartilage, adipocyte, fibroblast, marrow stroma, osteoclast- and hematopoietic-supportive stroma, cardiac muscle, smooth muscle, skeletal muscle, pericyte, vascular, epithelial, glial, neuronal, astrocyte or oligodendrocyte cell.

20 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Properties of STRO-1⁺ MACS-isolated cells co-labeled with anti-CD146 (CC9). (A) Sort region, R1, represents the double positive STRO-1^{BRT}/CD146⁺ population. (B) The incidence of clonogenic cell colonies (>50 cells) based
 25 on STRO-1^{BRT}/CD146⁺ expression was determined by limiting dilution analysis of 24 replicates per cell concentration using Poisson distribution analysis from 5 independent experiments. Forward (size) and perpendicular (granularity) light scatter characteristics of BMMNCs (C), STRO-1^{int}/CD146⁻ cells (D) and STRO-1^{BRT}/CD146⁺ cells (E). (F) RT-PCR analysis of STRO-
 30 1^{BRT}/CD146⁺ sorted marrow cells for CBFA1 (lane 2), osteocalcin (lane 4) and

GAPDH (lane 6) transcripts. Control cells (BMSSC cultures grown in the presence of dexamethasone) expressing CBFA1 (lane 1), osteocalcin (lane3), and GAPDH (lane 5) is also shown. Reaction mixes were subjected to electrophoresis on a 1.5% agarose gel and visualised by ethidium bromide staining. (G) *In situ* expression of CD146 on blood vessel (bv) walls (arrow) in human bone marrow (bm) sections near the bone (b) surface 20X. Sections were counter stained with Hematoxylin. (H) Dual Immunofluorescence staining demonstrating reactivity of the STRO-1 antibody labeled with Texas red and the CC9 antibody labeled with fluorescein isothiocyanate, reacting to blood vessel walls in frozen sections of human bone marrow.

Figure 2. Immunophenotypic analysis of DPSCs *in vivo*. The bar graph depicts the number of clonogenic colonies retrieved from single cell suspensions of dental pulp following immunomagnetic bead selection based on reactivity to antibodies that recognize STRO-1, CD146, and 3G5 and isotype-matched negative control antibodies. The data are expressed as the number of colony-forming units obtained in the bead positive cell fractions as a percentage of the total number of colonies in unfractionated pulp cells averaged from three separate experiments. Statistical significance (*) was determined using the student t-test (p 0.01) comparing the percent total number of colonies for each antibody with the corresponding isotype-matched control.

Figure 3. Reactivity of perivascular makers in dental pulp. (A) Immunolocalization of the STRO-1 antigen on blood vessels (small arrows) in human dental pulp (p) and around perineurium (large arrow) surrounding a nerve bundle (nb) 20X. (B) Dual Immunofluorescence staining demonstrating reactivity of the STRO-1 antibody labeled with Texas Red to dental pulp perineurium (arrow) in combination with an anti-neurofilament antibody labeled with fluorescein isothiosyanate staining the inner nerve bundle (nb), 40X. (C) Immunolocalization of the CD146 antigen to blood vessel walls in human

dental pulp tissue 20X. (D) Dual Immunofluorescence staining demonstrating reactivity of the STRO-1 antibody labeled with Texas red to a blood vessel and the CC9 antibody labeled with fluorescein isothiosyanate. (E) Immunohistochemical staining of pulp tissue with a rabbit polyclonal anti-DSP antibody (arrow) to the odontoblast outer layer (od). 20X. (F) 3G5 reactivity to a single pericyte (arrow) in a blood vessel (bv) wall 40X. Tissue sections were counter stained with Hematoxylin.

Figure 4. 3G5 reactivity to BMSSCs. (A) The representative histogram depicts a typical dual-color FACS analysis profile of whole bone marrow mononuclear cells (BMMNCs) expressing CD146 (PE) and 3G5 (FITC). (B) Colony efficiency assays were performed for all the different expression patterns observed (regions "R" 1-6). The data are expressed as the mean incidence of colony-forming units for each cell fraction averaged from three separate experiments.

Figure 5. Developmental potential of purified BMSSCs and DPSCs in vivo. Cytospin preparations of MACS/FACS isolated STRO-1^{BRT}/CD146⁺ marrow cells (arrow) stained with an antibody specific to α -smooth muscle actin (A) and von Willebrand Factor (B). CD146⁺ pulp cells (large arrow) isolated by immunomagnetic bead selection (magnetic beads depicted by small arrows), stained with an antibody specific to α -smooth muscle actin (C) and von Willebrand Factor. (D). (E) Ectopic bone formation (b) and haematopoietic/adipogenic marrow (bm) by *ex vivo* expanded cells derived from STRO-1^{BRT}/CD146⁺ BMSSCs transplanted with HA/TCP into immunocompromised mice for three months (E). (F) Ectopic formation of dentin (d) and fibrous pulp tissue (p) by *ex vivo* expanded cells derived from CD146⁺ DPSCs transplanted with HA/TCP into immunocompromised mice for three months. Sections were stained with Hematoxylin & Eosin.

- Figure 6 Expression of CD34, CD45 and Glycophorin-A on STRO-1 positive bone marrow mononuclear cells. Representative histograms depicting typical dual-colour flow cytometric analysis profiles of STRO-1 positive bone marrow mononuclear cells isolated initially by magnetic activated sorting and co-stained with antibodies directed against CD34 (A), CD45 (B) or Glycophorin-A (C). The STRO-1 antibody was identified using a goat anti-murine IgM-fluorescein isothiocyanate while CD34, CD45 and Glycophorin-A were identified using a goat anti-murine IgG- phycoerythrin. The high expressing STRO-1 fraction which contained the clonogenic MPC population was isolated by fluorescence activated cell sorting based on regions R1 and R2.
- Figure 7 Bone marrow MPC are STRO-1 bright, CD34 negative, CD45 negative and Glycophorin-A negative. The graph depicts the results of in vitro adherent colony formation assays performed for each of the different sorted STRO-1 bright populations selected by their co-expression or lack of either the CD34, CD45 or Gycophorin-A antigens, based on regions R1 and R2 as indicated in Figure 6. These data are expressed as the mean incidence of colony-forming units for each cell fraction averaged from two separate experiments.
- Figure 8 Reactivity of perivascular makers in different human tissues. Dual-colour immunofluorescence staining demonstrating reactivity of (A) STRO-1 and CD146, (B) STRO-1 and alpha-smooth muscle actin, and (C) 3G5 and CD146, on blood vessels and connective tissue present on spleen, pancreas (Panel 1), brain, kidney (Panel 2), liver, heart (Panel 3) and skin (Panel 4) 20X. The STRO-1 and 3G5 antibodies were identified using a goat anti-murine IgM-Texas Red while CD146 and alpha-smooth muscle actin were identified using a goat anti-murine or IgG-fluorescein isothiocyanate. Co-localization is indicated by overlapping areas of yellow and orange fluorescence (white arrows).

- Figure 9 Isolation of adipose-derived MPC by FACS. Representative flow cytometric histograms depicting the expression of STRO-1, CD146 and 3G5 in fresh preparations of peripheral adipose-derived single-cell suspensions generated following collagenase/dispase digestion as previously described (Shi and Gronthos 2003). The antibodies were identified using either a goat anti-murine IgM or IgG-phycoerythrin. Cell populations were then selected by FACS, based on their positivity (region R3) or negativity (region R2) to each marker and then plated into regular growth medium to assess the incidence of adherent colony-forming cells in each cell fraction.
- Figure 10 Clonogenic adipose-derived MPC are positive for STRO-1/3G5/CD146. The bar graph depicts the number of clonogenic colonies retrieved from single cell suspensions of enzymatically digested human peripheral adipose tissue, following fluorescence activated cell sorting, based on their reactivity to antibodies that recognize STRO-1, CD146, and 3G5 (Figure 9), then cultured in standard growth medium as previously described for bone marrow and dental pulp tissue (Shi and Gronthos 2003). The data are expressed as the number of colony-forming units obtained per 10^5 cells plated in the positive and negative cell fractions averaged from two separate experiments.
- Figure 11 Immunophenotypic analysis of adipose-derived MPC. Representative flow cytometric histograms depicting the co-expression of STRO-1 and CD146 (A) and 3G5 and CD146 in fresh preparations of peripheral adipose-derived single-cell suspensions generated following collagenase/dispase digestion. The STRO-1 and 3G5 antibodies were identified using a goat anti-murine IgM-phycoerythrin while CD146 was identified using a goat anti-murine IgG-fluorescein isothiocyanate. Approximately 60% and 50% of the CD146 positive cells co-express STRO-1 and 3G5, respectively. These data suggest that 10% or more of the CD164 positive cells co-express STRO-1 and 3G5.

- Figure 12 Developmental potential of purified Adipocyte-derived MPC *in vitro*. Preparations of primary MPC cultures derived from STRO-1⁺/CD146⁺ adipose cells were re-cultured either in standard culture conditions (A), osteogenic inductive medium (B), Adipogenic inductive medium (C) or chondrogenic conditions (D) as previously described Gronthos et al. 2003. Following two weeks of multi-differentiation induction, the adipocyte-derived MPC demonstrated the capacity to form bone (B; Alizarin positive mineral deposits), fat (C; Oil Red O positive lipid) and cartilage (D: collagen type II matrix).
- Figure 13 Isolation of skin-derived MPC by FACS. Representative flow cytometric histograms depicting the expression of STRO-1, CD146 and 3G5 in fresh preparations of full thickness skin-derived single-cell suspensions generated following collagenase/dispase digestion. The antibodies were identified using either a goat anti-murine IgM or IgG-phycoerythrin. Cell populations were then selected by FACS, based on their positivity (region R3) or negativity (region R2) to each marker and then plated into regular growth medium to assess the incidence of adherent colony-forming cells in each cell fraction.
- Figure 14 Clonogenic skin-derived MPC are positive for STRO-1/3G5/CD146. The bar graph depicts the number of adherent colonies recovered from single cell suspensions of enzymatically digested human skin, following fluorescence activated cell sorting, based on their reactivity to antibodies that recognize STRO-1, CD146, and 3G5 (Figure 6), then cultured in standard growth medium as previously described for bone marrow and dental pulp tissue (Shi and Gronthos 2003). The data are expressed as the number of colony-forming units obtained per 10⁵ cells plated in the positive and negative cell fractions averaged from two separate experiments.

Figure 15 A. Immunophenotypic expression pattern of ex vivo expanded bone marrow MPC. Single cell suspensions of ex vivo expanded bone marrow MPC were prepared by trypsin/EDTA treatment then incubated with antibodies identifying cell lineage-associated markers. For those antibodies identifying intracellular antigens, cell preparations were fixed with cold 70% ethanol to permeabilize the cellular membrane prior to staining for intracellular markers. Isotype matched control antibodies were treated under identical conditions. Flow cytometric analysis was performed using a COULTER EPICS instrument. The dot plots represent 5,000 listmode events indicating the level of fluorescence intensity for each lineage cell marker (bold line) with reference to the isotype matched negative control antibodies (thin line).

B. Gene expression profile of cultured MPC. Single cell suspensions of ex vivo expanded bone marrow MPC were prepared by trypsin/EDTA treatment and total cellular RNA was prepared. Using RNeasy extraction method total RNA was isolated and used as a template for cDNA synthesis, prepared using standard procedure. The expression of various transcripts was assessed by PCR amplification, using a standard protocol as described previously (Gronthos et al. 2003). Primers sets used in this study are shown in Table 2. Following amplification, each reaction mixture was analysed by 1.5% agarose gel electrophoresis, and visualised by ethidium bromide staining. Relative gene expression for each cell marker was assessed with reference to the expression of the house-keeping gene, GAPDH, using ImageQuant software.

Figure 16. *Ex vivo* expanded STRO-1^{br} MPC can develop into arterioles *in vitro*. Single cell suspensions of *ex vivo* expanded bone marrow STRO-1^{br} MPC were prepared by trypsin/EDTA treatment then plated into 48-well plates containing 200µl of matrigel. The STRO-1^{br} MPC were plated at 20,000 cells per well in serum-free medium (Gronthos et al. 2003) supplemented with the

growth factors PDGF, EGF, VEGF at 10ng/ml. Following 24 hours of culture at 37°C in 5% CO₂, the wells were washed then fixed with 4% paraformaldehyde. Immunohistochemical studies were subsequently performed demonstrated that the cord-like structures expressed alpha-smooth muscle actin identified with a goat-anti-murine IgG horse radish peroxidase antibody.

DETAILED DESCRIPTION OF THE ILLUSTRATED AND EXEMPLIFIED EMBODIMENTS OF THE INVENTION

10 The present invention relates to mesenchmal precursor cells, in particular those that may be present in the perivascular compartment of vascularised tissue. Such mesenchymal cells may be identified by the presence of the 3G5 surface marker, and perhaps additionally or separately by other early developmental markers such as CD146 (MUC18), VCAM-1 and STRO-1.

15 Precursor cells are early cells that are substantially at a pre-expansion stage of development. These are cells that have yet to differentiate to fully committed cells, however they need not be stem cells in a strict sense, in that they are necessarily able to differentiate into all types of cells. Partially differentiated precursor cells have a benefit in that they have a greater
20 proliferative potential than stem cells.

The present precursor cells are somewhat differentiated in that they are committed to mesenchymal tissue, as opposed, for example, to haemopoietic tissues. It is evident from the data produced that the MPCs that have been isolated lack markers associated with
25 haemopoietic cells such as CD34, and additionally their differentiation potential does not extend to haemopoietic lines. Additionally they need not necessarily have the potential to differentiate into all mesenchymal cell type, rather, they may be able to differentiate into one, two three or more cell types.

It is anticipated that these precursor cell harvested from the tissues concerned may be useful for regenerating tissue for cells types from which they have been sourced. Thus precursor cells isolated from heart may be reintroduced to regenerate heart tissue, however their potential need not be so limited, precursor cells isolated from one tissue type might be useful
5 for regenerating tissue in another tissue type. The microenvironment in which an undifferentiated cell finds itself is known to exert an influence on the route of differentiation and therefore the reintroduction need not necessarily be tissue specific.

The data presented show that MPCs have been harvested and then re-introduced to produce
10 bone and bone marrow and dentin and pulp respectively, in addition arterioles, cord like structures, have been produced after ex vivo expansion of isolated MPCs.

It is anticipated that a wide range of cells might be produced based on gene expression of markers characteristic for certain cell types. It is thus anticipated that under appropriate
15 culture conditions the range of cell types that can be generated from the perivascular MPCs of the present invention include but are not limited to the following, osteoblast, odontoblast, dentin-producing, chondrocyte, tendon, ligament, cartilage, adipocyte, fibroblast, marrow stroma, osteoclast- and hematopoietic-supportive stroma, cardiac muscle, smooth muscle, skeletal muscle, pericyte, vascular, epithelial, glial, neuronal, astrocyte or oligodendrocyte
20 cell.

One of the benefits of the finding that MPCs can be isolated from perivascular cells is that this greatly expands the range of source tissues from which MPCs can be isolated or enriched and there is no longer an effective restriction on the source of MPCs to bone marrow. The
25 tissues from which these MPCs have been isolated in the exemplifications of this invention are human bone marrow, dental pulp cells, adipose tissue and skin. In addition in situ staining and histological studies have identified that MPC are present in the perivascular compartment of spleen, pancreas, brain, kidney, liver and heart. Given this wide and diverse range of tissue types where perivascular MPCs are present, it is proposed that MPC will also
30 be present from an even wider range of tissue which may include, adipose tissue, teeth,

dental pulp, skin, liver, kidney, heart, retina, brain, hair follicles, intestine, lung, spleen, lymph node, thymus, pancreas, bone, ligament, bone marrow, tendon, and skeletal muscle.

These precursor cells of the present invention are distinguished from other known MPCs in that they are positive for 3G5 or perhaps that they carry another perivascular markers. They can be isolated by enriching for an early developmental surface marker present on perivascular cells, in particular the presence of one or more of CD146(MUC18), VCAM-1 and alternatively or additionally high level expression of the marker recognised by the monoclonal antibody STRO-1. Alternatively or additionally enrichment may be carried out using 3G5.

Markers associated with perivascular cells may also be present on the MPCs, for example alpha smooth muscle actin (α SMA).

Other early developmental markers associated with MPCs may also be present. These may include but are not necessarily limited to the group consisting of THY-1, VCAM-1, ICAM-1, PECAM-1, CD49a/CD49b/CD29, CD49c/CD29, CD49d/CD29, CD29, CD61, integrin beta 5, 6-19, thrombomodulin, CD10, CD13, SCF, STRO-1bri, PDGF-R, EGF-R, IGF1-R, NGF-R, FGF-R, Leptin-R (STRO-2). Positive expression of one or more of these markers may be used in methods of enriching for MPCs from source tissue.

The MPCs of the present invention may also be characterised by the absence of markers present in differentiated tissue, and enrichment may be based on the absence of such markers.

Similarly it is preferred that the enriched cell populations are not of haemopoietic origin and thus it is preferred that these cells are not present. Markers characteristically identified as not present include but are not limited to CD34, CD45 and glycophorin A. Additional other markers for this purpose might include CD20 and CD19 (B lymphocyte markers), CD117 (c-

kit oncoprotein) present on hemopoietic stem cells and angioblasts, CD14 (macrophage), CD3 and CD4 (T cells).

It may be desirable to use the relatively quiescent, directly enriched or isolated perivascular
5 MCPs. Alternatively it has been discovered that expansion of the enriched population can be carried out and have the beneficial effect of resulting in much greater numbers of cells. The effect of expansion of the directly enriched pool of cells is, however, that some differentiation of the initial MCPs will occur. Expansion over a 5 week period might result in an increase of 10^3 fold. Other periods might be chosen to expand the population to between
10 10^2 to 10^5 fold. This potential might be directed by culturing them in media containing cytokines and other factors directing the differentiation to a particular tissue type for example PDGF and VEGF forming smooth muscle alpha cords. These could then be introduced into a tissue with, for example, an insult to assist with repair. Alternatively it may be desired after expansion to re select cells on the basis of an early developmental marker, that might be
15 STRO-1^{bni} to increase the proportion of MPCs in the population.

It is found that an essentially pure population of MCPs is not necessary to provide for formation of differentiated cells to form desired tissue structures. The enriched population may have levels of MCPs of greater than about 0.001, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 or 1% or
20 higher as a proportion of total cell numbers in the enriched population. This order of enrichment can be achieved by the use of a single marker for selection of the enriched MCP population. This is particularly so where the source tissue has an inherently high level of perivascular MCPs. It is found that considerably more 3G5 pos MCPs are present in certain tissue, for example dental pulp, than in bone marrow. Thus in bone marrow 3G5 positive
25 MPCs constitute about 15% of MPC based on STR1^{bni} colony forming cells, whereas in dental pulp that are found to constitute 65% and greater than 90% in fat and skin tissues. Expansion of the population and then re-enrichment using a single marker could result in higher levels of MPCs, perhaps levels greater than about 0.1, 0.5, 1, 2, 5 or 10%

Whilst it is considered desirable that a substantial proportion and preferably a majority of precursor cells are perivascular MPCs, it is not considered essential for certain forms of the invention for perivascular MPCs to be the sole precursor cell form. Other forms of precursors may also be present without unduly interfering with the capacity of the perivascular MPCs to undergo the desired differentiation. Such other forms may include haemopoietic precursors or non-perivascular MPCs, perhaps being negative for 3G5.

Certain forms of the present invention provide perivascular MPCs substantially free of endothelial cells. In that context substantially free might be considered to be less than about 5, 2, 1, or 0.1% endothelial cells. Alternatively the context might be an assessment that the enriched population is von Willebrand Factor negative.

It will be understood that recognition of cells carrying the cell surface markers that form the basis of the separation can be effected by a number of different methods, however, all of these methods rely upon binding a binding agent to the marker concerned followed by a separation of those that exhibit binding, being either high level binding, or low level binding or no binding. The most convenient binding agents are antibodies or antibody based molecules, preferably being monoclonal antibodies or based on monoclonal antibodies because of the specificity of these latter agents. Antibodies can be used for both steps, however other agents might also be used, thus ligands for these markers may also be employed to enrich for cells carrying them, or lacking them.

The antibodies may be attached to a solid support to allow for a crude separation. The separation techniques should maximise the retention of viability of the fraction to be collected. Various techniques of different efficacy may be employed to obtain relatively crude separations. The particular technique employed will depend upon efficiency of separation, associated cytotoxicity, ease and speed of performance, and necessity for sophisticated equipment and/or technical skill. Procedures for separation may include, but are not limited to, magnetic separation, using antibody-coated magnetic beads, affinity chromatography and "panning" with antibody attached to a solid matrix. Techniques providing accurate separation include but are not limited to FACS.

It is in the context of these methods that a cell be either negative or positive. The positive cells may either be low(lo) or a hi (bright) expresser depending on the degree to which the marker is present on the cell surface, the terms relate to intensity of fluorescence or other color used in the color sorting process of the cells. The distinction of lo and hi will be understood in the context of the marker used on a particular cell population being sorted.

The method of enriching for perivascular MPCs might include the step of making a first partially enriched pool of cells by enriching for the expression of a first of the markers, and then the step of enriching for expression of the second of the markers from the partially enriched pool of cells.

It is preferred that the method comprises a first step being a solid phase sorting step, based on recognition of one or more of the markers. The solid phase sorting step of the illustrated embodiment utilises MACS recognising high level expression of STRO-1. This then gives an enriched pool with greater numbers of cells than if a high accuracy sort was used as a first step. If for example FACS is used first, many of the precursor cells are rejected because of their association with other cells. A second sorting step can then follow using an accurate separation method. This second sorting step might involve the use of two or more markers. Thus in the illustrated embodiment two colour FACS is used to recognise high level expression of the antigen recognised by STRO-1 as well as the expression of CD146. The windows used for sorting in the second step can be more advantageously adjusted because the starting population is already partially enriched.

The method of enriching for perivascular MPCs might also include the harvesting of a source of the stem cells before the first enrichment step using known techniques. Thus the tissue will be surgically removed. Cells comprising the source tissue will then be separated into a so called single cells suspension. This separation may be achieved by physical and or enzymic means.

The preferred source of such perivascular MPCs is human, however, it is expected that the invention is also applicable to animals, and these might include agricultural animals such as cows, sheep, pigs and the like, domestic animals such as dogs, laboratory animals such as mice, rats, hamsters, and rabbits or animals that might be used for sport such as horses.

entombed by mineralized dentin.⁽¹¹⁻¹³⁾ Following tooth maturation, dental pulp becomes relatively static, acting only in a reparative capacity in response to a compromised dentin matrix caused by insults such as caries or mechanical trauma.

5 Precursors of functional osteoblasts (BMSSCs: bone marrow stromal stem cells) and odontoblasts (DPSCs: dental pulp stem cells), both forms of MPCs identified by their source tissue, were initially identified by their capacity to form clonogenic cell clusters *in vitro*, a common feature amongst different stem cell populations.^(4,14-18) The progeny of *ex vivo* expanded BMSSCs and DPSCs share a similar gene expression profile for a variety of
10 transcriptional regulators, extracellular matrix proteins, growth factors/receptors, cell adhesion molecules, and some, but not all lineage markers characteristic of fibroblasts, endothelial cells, smooth muscle cells and osteoblasts.^(4,19) However, previous studies have documented that individual BMSSC colonies demonstrate marked differences in their proliferation rates *in vitro* and developmental potentials *in vivo*.^(5,14,20) Similar to these
15 findings, we have recently observed comparable levels of heterogeneity in the growth and developmental capacity of different DPSC colonies.⁽²¹⁾ Together, these studies infer a hierarchical arrangement of stromal precursor cells residing in bone marrow and dental pulp, headed by a minor population of highly proliferative pluri-potential stem cells that give rise to committed bi- and uni-potential progenitor cell populations.⁽²²⁾
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Despite our extensive knowledge about the properties of cultured BMSSCs and DPSCs, we still do not know if their *in vitro* characteristics are an accurate portrait of their true gene expression patterns and developmental potentials *in situ*. In addition, it is not formally known if all of the colony-forming cells within each tissue are derived from one pluri-potent
25 stem cell pool or whether they arise from committed progenitors belonging to distinct lineages. There is also a lack of information regarding the precise anatomical location of BMSSCs and DPSCs in their respective tissues. This is mainly attributed to the rarity of stem cells and the absence of specific markers that identify different developmental stages during osteogenesis and odontogenesis, particularly for primitive subpopulations. It has previously

been hypothesized that one possible niche for precursors of osteoblasts and odontoblasts may be the microvasculature networks of bone marrow and dental pulp, respectively.^(23,24)

MATERIALS AND METHODS

5 *Tissue Samples*

Iliac crest-derived bone marrow mononuclear cells (BMMNCs), from normal human adult volunteers were obtained under guidelines set by the Royal Adelaide Hospital Human Ethics Committee. Normal human impacted third molars were collected from young adults the University of Adelaide Dental Clinic Research under approved guidelines set by the
10 University of Adelaide Human Ethics Committee, respectively. Discarded full thickness skin and peripheral adipose tissue were obtained from routine plastic surgery procedures from the Skin Cell Engineering Laboratory, under the guidelines set by the Royal Adelaide Hospital Human Ethics Committee. The pulp tissue was separated from the crown and root as previously described.⁽⁴⁾ Single cell suspensions of dental pulp, skin and adipose tissue were
15 prepared by enzymatic digestion in a solution of 3 mg/ml collagenase type I (Worthington Biochem, Freehold, NJ) and 4 mg/ml dispase (Boehringer Mannheim, GMBH, Germany) for one to three hours at 37°C. Single cell suspensions were obtained by passing the cells through a 70 µm strainer (Falcon, BD Labware, Franklin Lakes, NJ). Cell (0.01 to 1 x 10⁵/well) preparations of bone marrow, dental pulp, skin and adipose were then used for
20 either, immunolselection, RNA extraction, or direct culture in 6-well plates (Costar, Cambridge, MA) as described below.

Other human tissue specimens (Brain, liver, heart , kidney, lung, spleen, thymus, lymph node, pancreas, skin) were obtained from autopsies carried out at the Royal Adelaide Hospital
25 during routine pathological examinations under approved guidelines set by the Royal Adelaide Hospital Human Ethics Committee. Small specimens approximately 0.5 cm² of each tissue type were placed into Tissue-Tek II cryomoulds 25 mm x 20 mm x 5 mm (Miles Laboratories; Naperville, IL) and embedded with O.C.T. compound medium (Miles Laboratories) by immersion into a 150ml to 200ml pyrex glass beaker of iso-pentane (BDH

Chemicals, Poole, England) pre-cooled by suspending a glass beaker into a bath of liquid nitrogen. The isopentane has cooled when the bottom of the glass is white. The frozen sections were immediately stored at -80°C. Frozen sections of nerve and muscle tissue were obtained from the Histopathology Department of the I.M.V.S., South Australia and sections of
5 foreskin were obtained from the Immunology Department of the I.M.V.S., South Australia. Sections of formalin fixed, paraffin embedded human foetal limb (52 days) were kindly provided by Dr. T.J. Khong from the Department of Histopathology, Women's and Children's Hospital, Adelaide, South Australia.

10 *Colony Efficiency Assay and Culture*

Single cell suspensions were plated at low plating densities (between 1,000 and 10,000 cells per well, as triplicates in six well plates) to assess colony-forming efficiency of different immunoselected cell fractions. The cells were cultured in alpha-Modification of Eagle's Medium supplemented with 20% foetal calf serum, 2mM L-Glutamine, 100 µM L-ascorbate-
15 2-phosphate, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO₂. Day 14 cultures were fixed with 4% formalin, and then stained with 0.1% toluidine blue. Aggregates of equal to or greater than fifty cells were scored as clonogenic colonies equivalent to colony forming units-fibroblastic (CFU-F).

20 *Magnetic-Activated Cell Sorting (MACS)*

This procedure is a modification of that described elsewhere.⁽²⁵⁾ Briefly, approximately 1 x 10⁸ BMMNCs were incubated with STRO-1bri supernatant (murine anti-human BMSSCs, IgM)⁽²⁹⁾ (1/2) for 1 hour on ice. The cells were then washed with PBS/5% FBS and resuspended in a 1/50 dilution of biotinylated goat anti-mouse IgM (µ-chain specific; Caltag
25 Laboratories, Burlingame, CA) for 45 minutes on ice. After washing, the cells were incubated with streptavidin microbeads (Miltenyi Biotec, Bergisch Gladbach, F.R.G.) for 15 minutes on ice, then separated on a Mini MACS magnetic column (Miltenyi Biotec) according to the manufacturers recommendations.

Fluorescence activated Cell Sorting (FACS)

STRO-1^{bri} MACS isolated cells were incubated with a streptavidin-FITC conjugate (1/50; CALTAG Laboratories) for 20 minutes on ice then washed with PBS/5% FBS. Single-color
5 fluorescence activated cell sorting (FACS) was performed using a FACStar^{PLUS} flow
cytometer (Becton Dickinson, Sunnyvale, CA). Dual color-FACS analysis was achieved by
incubating MACS-isolated STRO-1^{bri} BMMNCs with saturating (1:1) levels of CC9
antibody supernatant (mouse anti-human CD146/MUC-18/Mel-CAM, IgG_{2a}, Dr. Stan
Gronthos) for one hour on ice. After washing with PBS/5% FBS, the cells were incubated
10 with a second label goat anti-mouse IgG_{2a} (γ-chain specific) phycoerythrin (PE) conjugate
antibody (1/50, CALTAG Laboratories) for 20 minutes on ice. The cells were then sorted
using the automated cell deposition unit (ACDU) of a FACStar^{PLUS} flow cytometer. Limiting
dilution assay: seeded 1, 2, 3 4, 5, & 10 cells per well, 24 replicates, cultured in serum-
deprived medium for 10 days as previously described ⁽²⁶⁾. Similarly, freshly prepared
15 unfractionated BMMNCs were incubated with CC9 (IgG_{2a}) and 3G5 (IgM) antibodies or
isotype-matched negative control antibodies for one hour on ice. After washing with
PBS/5% FBS, the cells were incubated with a second label goat anti-mouse IgG_{2a} (γ-chain
specific) phycoerythrin (PE) and IgM (1/50; CALTAG Laboratories) conjugated antibodies
for 30 minutes on ice. Cells were washed in PBS/5% FBS prior to being analysed using a
20 FACStar^{PLUS} flow cytometer. Positive reactivity for each antibody was defined as the level
of fluorescence greater than 99% of the isotype matched control antibodies.

Flow Cytometric Analysis

Single cell suspensions of ex vivo expanded bone marrow MPC were prepared by
25 trypsin/EDTA treatment then incubated with neat STRO-1 supernatant or antibodies
identifying different cell lineage-associated markers (10 μg/ml) for one hour on ice. The cells
were then washed in PBS/5% FBS then incubated either with a goat anti-murine IgM-
phycoerythrin (1/50, SouthernBiotechnologies), goat anti-murine or anti-rabbit IgG-
phycoerythrin (Caltag Laboratories). For those antibodies identifying intracellular antigens,

cell preparations were permeabilize the cellular membrane prior to staining for intracellular markers. Isotype matched control antibodies were treated under identical conditions. Flow cytometric analysis was performed using a COULTER EPICS instrument. The dot plots represent 5,000 listmode events indicating the level of fluorescence intensity for each lineage cell marker with reference to the isotype matched negative control antibodies.

Immunohistochemistry

Human tissue sections (μm) were de-waxed in xylene and rehydrated through graded ethanol into PBS. Frozen tissue sections (μm) and cytospin preparations were fixed with cold acetone at -20°C for 15 minutes then washed in PBS. The samples were subsequently treated with PBS containing 1.5% of hydrogen peroxide for 30 minutes, washed then blocked with 5% non-immune goat serum for 1 hour at room temperature. Samples were incubated with primary antibodies for 1 hour at room temperature. Antibodies used: Mouse (IgG_1 & IgG_{2a}) controls (Caltag, Burlingame, CA); Rabbit (Ig) control, 1A4 (anti- α smooth muscle actin, IgG_1), 2F11 (anti-neurofilament, IgG_1), F8/86 (murine anti-von Willebrand Factor, IgG_1) (Dako, Carpinteria, CA); STRO-1; CC9 (anti-CD146); LF-151 (rabbit anti-human dentinsialoprotein; Dr. L. Fisher, NIDCR/NIH, MD). Working dilutions: rabbit serum (1/500), monoclonal supernatants (1/2) and purified antibodies (10 $\mu\text{g}/\text{ml}$). Single staining was performed by incubating the samples with the appropriate secondary antibody, biotinylated goat anti-mouse IgM, IgG_1 , IgG_{2a} or biotinylated goat anti-rabbit for one hour at room temperature (Caltag Laboratories). Avidin-Peroxidase-complex and substrate were then added according to the manufacturer instructions (Vectastain ABC Kit standard, Vector Laboratories). Samples were counterstained with hematoxylin and mounted in aqueous media. Dual-fluorescence labeling was achieved by adding the secondary antibodies, goat anti-mouse IgM-Texas Red and IgG-FITC (CALTAG Laboratories), for 45 minutes at room temperature. After washing the samples were mounted in VECTASHIELD fluorescence mountant.

Immunomagnetic bead selection

Single cell suspensions of dental pulp tissue were incubated with antibodies reactive to STRO-1 (1/2), CD146 (1/2), or 3G5 (1/2) for 1 hour on ice. The cells were washed twice with PBS/1%BSA then incubated with either sheep anti-mouse IgG-conjugated or rat anti-mouse IgM-conjugated magnetic Dynabeads (4 beads per cell: Dynal, Oslo, Norway) for 40 minutes on a rotary mixer at 4°C. Cells binding to beads were removed using the MPC-1 magnetic particle concentrator (Dynal) following the manufactures recommended protocol.

Matrigel-Arteriole Assay

Single cell suspensions of ex vivo expanded bone marrow STRO-1^{bright} MPC were prepared by trypsin/EDTA treatment then plated into 48-well plates containing 200µl of matrigel. The STRO-1^{bright} MPC were plated at 20,000 cells per well in serum-free medium (Gronthos et al. 2003) supplemented with the growth factors PDGF, EGF, VEGF at 10ng/ml. Following 24 hours of culture at 37°C in 5% CO₂, the wells were washed then fixed with 4% paraformaldehyde. Immunohistochemical studies were subsequently performed for alpha-smooth muscle actin identified with a goat-anti-murine IgG horse radish peroxidase antibody/Vectastaining Kit as described above.

Osteogenic, Adipogenic and Chondrogenic Differentiation of MPC in vitro

Single cell suspensions of ex vivo expanded adipose-derived MPC were cultured in αMEM supplemented with 10% FCS, 100 µM L-ascorbate-2-phosphate, dexamethasone 10⁻⁷ M and 3 mM inorganic phosphate previously shown to induce bone marrow MPC to form a mineralized bone matrix in vitro (Gronthos et al., 2003). Mineral deposits were identified by positive von Kossa staining. Adipogenesis was induced in the presence of 0.5 mM methylisobutylmethylxanthine, 0.5 µM hydrocortisone, and 60 µM indomethacin as previously described (Gronthos et al. 2003). Oil Red O staining was used to identify lipid-laden fat cells. Chondrogenic differentiation was assessed in aggregate cultures treated with 10 ng/ml TGF-β3 as described (Pittenger et al., 1999)

In vivo transplantation studies

Approximately 5.0×10^6 of *ex vivo* expanded cells derived from either STRO-1^{bri}/CD146⁺ BMSSCs or CD146⁺ DPSCs were mixed with 40 mg of hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (Zimmer Inc, Warsaw, IN) and then transplanted subcutaneously into the dorsal surface of 10-week-old immunocompromised beige mice (NIH-bg-nu-xid, Harlan Sprague Dawley, Indianapolis, IN) as previously described.⁽⁴⁾ These procedures were performed in accordance to specifications of an approved animal protocol (NIDCR #00-113).

Reverse transcription-polymerase chain reaction.

Total RNA was prepared from STRO-1^{BRT}/CD146⁺ sorted BMMNCs, and control cells (primary BMSSC cultures grown in the presence of 10^{-7} M dexamethasone for three weeks) using RNA STAT-60 (TEL-TEST Inc. Friendswood TX). First-strand cDNA synthesis was performed with a first-strand cDNA synthesis kit (GIBCO BRL, Life Technologies) using an oligo-dT primer. First strand cDNA (2 μ l) was added to 46 μ l of a 1X PCR master reaction mix (Roche Diagnostics, GmbH Mannheim Germany) and 10 pMol of each human specific primer sets: CBFA1 (632bp, and three smaller alternative splice variants)⁽²⁷⁾ sense 5'-CTATGGAGAGGACGCCACGCCTGG-3' [SEQ ID NO. 1], antisense, 5'-CATAGCCATCGTAGCCTTGTCT-3' [SEQ ID NO. 2]; osteocalcin (310bp)⁽⁴⁾ sense, 5'-CATGAGAGCCCTCACA-3' [SEQ ID NO. 3], antisense, 5'-AGAGCGACACCCTAGAC-3' [SEQ ID NO. 4]; GAPDH (800bp)⁽⁴⁾ sense, 5'-AGCCGCATCTTCTTTTTCGTC-3' [SEQ ID NO. 5]; antisense 5'-TCATATTTGGCAGGTTTTTCT-3' [SEQ ID NO. 6]. The reactions were incubated in a PCR Express Hybaid thermal cycler (Hybaid, Franklin, MA) at 95°C for 2 minutes for 1 cycle then 94°C/(30 sec), 60°C/(30 sec), 72°C/(45 sec) for 35 cycles, with a final 7 minute extension at 72°C. Following amplification, each reaction was analyzed by 1.5% agarose gel electrophoresis, and visualized by ethidium bromide staining.

RESULTS

BMSSCs and DPSCs express vascular associated antigens STRO-1 and CD146 in vivo.

We have previously demonstrated the efficacy of magnetic activated cell sorting (MACS), to isolate and enrich for all detectable clonogenic colonies from aspirates of human marrow, based on their high expression of STRO-1 antigen.^(25,26) To further characterize BMSSCs we incubated the STRO-1^{bri} MACS isolated cells with another monoclonal antibody, CC9,⁽²⁸⁾ that recognizes the cell surface antigen CD146, also known as MUC-18, Mel-CAM and Sendo-1, that is present on endothelial and smooth muscle cells. These studies determined that CC9, selectively bound the STRO-1 bright expressing fraction (STRO-1^{BRT}) from the total STRO-1⁺ population by dual-color FACS analysis (Figure 1A). Cloning efficiency assays using Poisson distribution statistics, yielded a marked increase in the incidence of BMSSCs (1 colony per 5 STRO-1^{BRT}/CD146⁺ cells plated), and achieved a 2×10^3 fold enrichment of the clonogenic colony population when compared to unfractionated marrow (Figure 1B). No colony formation could be detected in STRO-1^{BRT}/CD146⁻ cell fraction (data not shown).

The light scatter properties of STRO-1^{BRT}/CD146⁺ marrow cells were typically larger and more granular than the nucleated erythroid cells and B-lymphocytes comprising the bulk of the STRO-1⁺ population⁽²⁹⁾ (Figure 1C-E). Cytospin preparations of STRO-1^{BRT}/CD146⁺ sorted cells were found to be negative for the erythroid (glycophorin-A) and leukocyte (CD45) associated markers (data not shown). Confirmation that BMSSCs represented an early osteogenic precursor population was obtained by RT-PCR analysis of highly purified MACS/FACS-isolated STRO-1^{BRT}/CD146⁺ cells, which failed to detect the early and late osteogenic, markers CBFA1 and osteocalcin, respectively (Figure 1F). However, the progeny of STRO-1^{BRT}/CD146⁺ sorted BMSSCs were found to express both CBFA1 and osteocalcin, following *ex vivo* expansion. Immunolocalization studies demonstrated that the CD146 antigen was predominantly expressed on blood vessel walls in sections of human bone marrow (Figure 1G). Localization of both STRO-1 and CD146 was confined to large blood vessels in frozen sections of human bone marrow trephine (Figure 1H).

30

Immunoselection protocols were subsequently used to determine if human DPSCs also expressed STRO-1 and CD146 *in situ*. The use of either MACS or FACS analysis to isolate DPSCs was restrictive due to the rarity of these cells (1 colony-forming cell per 2×10^3 cells plated) compounded by the limited number of pulp cells (approximately 10^5 cells per pulp sample) obtained following processing. To circumvent this, we pooled several pulp tissues obtained from 3 to 4 different third molars per experiment and employed immunomagnetic bead selection on single cell suspensions of pulp tissue, based on their expression of either the STRO-1 or CD146 antigens. The STRO-1⁺ fraction represented approximately 6% of the total pulp cell population. Comparative studies demonstrated that growth rates of individual colonies were unperturbed in the presence of magnetic beads (data not shown). Colony efficiency assays indicated that the majority of dental pulp derived colony-forming cells (82%) were represented in the minor, STRO-1⁺ cell fraction analogous to BMSCs (Figure 2). The mean incidence of DPSCs in the STRO-1 positive fraction (329 colony-forming cells per 10^5 cells plated \pm 56 SE, n=3) was six-fold greater than unfractionated pulp cells (55 colony-forming cells per 10^5 cells plated \pm 14 SE, n=3). Using a similar strategy, different fractions of human dental pulp cells were selected based on their reactivity with the antibody, CC9. Colony efficiency assays showed that a high proportion (96%) of dental pulp-derived clonogenic colonies were also present in the CD146⁺ population, using immunomagnetic Dynal bead selection (Figure 2). The mean incidence of clonogenic colonies in the CD146⁺ fraction (296 colony-forming cells per 10^5 cells plated \pm 37 SE, n=3) was seven-fold greater than unfractionated pulp cells (42 colony-forming cells per 10^5 cells plated \pm 9 SE, n=3).

Immunolocalization studies showed that STRO-1 expression was restricted to blood vessel walls and perineurium surrounding the nerve bundles, but was not present in the mature odontoblast layer or fibrous tissue, in frozen sections of human dental pulp tissue (Figure 3A-B). Furthermore, co-localization of CD146 with STRO-1 was detected on the outer blood vessel cell walls, with no reactivity to the surrounding fibrous tissue, odontoblast layer, and the perineurium of the nerve (Figure 3C-D). Importantly, expression of human odontoblast-specific differentiation marker, dentinsialoprotein (DSP), was restricted to the

outer pulpal layer containing mature odontoblasts (Figure 3E) and was absent in fibrous tissue, nerve bundles and blood vessels.

Differential expression of the perivascular marker 3G5 by BMSSCs and DPSCs.

5 In the present study, flow cytometric analysis revealed that the cell surface antigen, 3G5, was highly expressed by a large proportion (54%) of hematopoietic marrow cells (Figure 4A). This observation eliminated 3G5 as a candidate marker for isolating purified populations of BMSSCs directly from aspirates of human marrow. In addition, dual-FACS analysis based on 3G5 and STRO-1 expression was not possible since both antibodies shared the same
10 isotype. Nevertheless, *in vitro* colony efficiency assays for different 3G5/CD146 FACS sorted subfractions demonstrated that only a minor proportion (14%) of bone marrow clonogenic colonies expressed the 3G5 antigen at low levels (Figure 4B). Conversely, a larger proportion (63%) of clonogenic DPSCs (192 colony-forming cells per 10^5 cells plated ± 18.4 SE $n=3$) were present in the 3G5⁺ cell fraction following immunomagnetic bead
15 selection (Figure 2). 3G5 demonstrated specific reactivity to pericytes in frozen sections of human dental pulp tissue (Figure 3F).

We next analyzed the expression of more specific markers of endothelial cells (von Willebrand Factor) and smooth muscle cells/pericytes (α -smooth muscle actin) on cytospin
20 preparations using freshly isolated STRO-1^{BRT}/CD146⁺ BMSSCs and CD146⁺ expressing DPSCs. A large proportion of purified BMSSCs (67%), were found to be positive for α -smooth muscle actin (Figure 5A), but lacked expression of von Willebrand Factor (Figure 5B). Similarly, the majority of isolated DPSCs (85%) were also found to express α -smooth muscle actin, but not von Willebrand Factor (Figure 5C, 5D). Purified populations of STRO-
25 1^{BRT}/CD146⁺ BMSSCs and CD146⁺ DPSCs were subsequently expanded *in vitro* then transplanted into immunocompromised mice to assess their developmental potentials *in vivo*. The progeny of cultured BMSSCs and DPSCs displayed distinct capacities, capable of regenerating the bone marrow and dental/pulp microenvironments, respectively (Figure 5E,

F), and appeared identical to the developmental potential of non-selected multi-colony derived BMSSCs and DPSCs (4).

DISCUSSION

- 5 The present study provides direct evidence that two mesenchymal stem cell populations, distinct in their ontogeny and developmental potentials, are both associated with the microvasculature of their respective tissues.

We employed different immunoselection protocols to demonstrate that BMSSCs and DPSCs
10 could be efficiently retrieved from bone marrow aspirates and enzyme digested pulp tissue respectively, based primarily on their high expression of the STRO-1 antigen. This cell surface antigen is present on precursors of various stromal cell types including, marrow fibroblasts, osteoblasts, chondrocytes, adipocytes, and smooth muscle cells isolated from human adult and fetal bone marrow.^(29,32-34) Previous studies have implicated STRO-1 as a
15 marker of pre-osteogenic populations, where its expression is progressively lost following cell proliferation and differentiation into mature osteoblasts *in vitro*.^(27,35,36) The STRO-1 antigen was also found to be present on the outer cell walls of human bone marrow and dental pulp blood vessels, in accord with previous studies that localized STRO-1 on large blood vessels, but not capillaries, in different adult tissues such as brain, gut, heart, kidney,
20 liver, lung, lymphnode, muscle, thymus.⁽⁶⁾ Therefore, STRO-1 appears to be an early marker of different mesenchymal stem cell populations and infers a possible perivascular niche for these stem cell populations *in situ*.

To determine if BMSSCs and DPSCs were associated directly with blood vessels we utilized
25 another antibody (CC9),⁽²⁸⁾ which recognizes the immunoglobulin super family member, CD146 (MUC-18/Mel-CAM), known to be present on smooth muscle, endothelium, myofibroblasts and Schwann cells *in situ*, as well as being a marker for some human neoplasms.⁽³⁷⁾ Notably, CD146 is not expressed by bone marrow hematopoietic stem cells, nor their progenitors. While the precise function of CD146 is not known, it has been linked

to various cellular processes including cell adhesion, cytoskeletal reorganization, cell shape, migration and proliferation through transmembrane signaling.

In order to dissect the BMSSC population, STRO-1^{BRT} expressing marrow cells were further distinguished from STRO-1⁺ hematopoietic cells (predominantly glycophorin-A⁺ nucleated erythrocytes) based on their expression of CD146, using dual-FACS analysis. Purified STRO-1^{BRT}/CD146⁺ human BMSSCs displayed light scatter properties characteristic of large granular cells. Our study supports the findings of Van Vlasselaer and colleagues (1994)⁽³⁸⁾ who isolated partially purified BMSSCs from murine bone marrow following 5-fluoracil (5-FU) treatment, and identified this population as having high perpendicular and forward light scatter characteristics. Interestingly, freshly isolated 5-FU resistant murine BMSSCs were also found to be positive for two perivascular markers Sab-1 and Sab-2.⁽³⁸⁾ Conversely, more recent studies have shown that when BMSSCs are cultivated *in vitro*, the most primitive populations display low perpendicular and forward light scatter properties⁽³⁹⁾ and therefore may not reflect the true morphology of BMSSC *in situ*. In the present study, STRO-1^{BRT}/CD146⁺ sorted human BMSSCs lacked the expression of CBFA1 and osteocalcin that identify committed early and late osteogenic populations, respectively,^(40,41) indicating that BMSSCs exhibit a pre-osteogenic phenotype in human bone marrow aspirates. We found that a high proportion of freshly isolated STRO-1^{BRT}/CD146⁺ BMSSCs expressed α -smooth muscle actin, but not the endothelial specific marker von Willebrand Factor, providing direct evidence that this primitive precursor population displays a characteristic perivascular phenotype.

The present study also demonstrated the efficacy of using magnetic bead selection to isolate and enrich for DPSCs directly from human dental pulp tissue based on their expression of either STRO-1 or CD146. Immunolocalization of CD146 appeared to be specific to the microvasculature within dental pulp. Co-localization of both STRO-1 and CD146 on the outer walls of large blood vessel in dental pulp tissue, implied that the majority of DPSCs arise from the microvasculature. However, since the STRO-1 antibody also reacted with the

perineurium in dental pulp and peripheral nerve bundles (unpublished observations), further investigation is required to determine the role of this antigen in neural cell development.

Analogous to BMSSCs, freshly isolated CD146⁺ DPSCs were found to express α -smooth muscle actin but not von Willebrand Factor. DPSCs were also shown to be an immature pre-odontogenic population both by their location distal from the dentin forming surface and by their lack of expression of the human odontoblast-specific dentin sialoprotein (DSP), which is restricted to the outer pulpal layer containing differentiated odontoblasts. We have previously described that *ex vivo* expanded human DPSCs do not express the precursor molecule, dentinsialophosphoprotein (DSPP), *in vitro* when cultured under non-inductive conditions.⁽⁴⁾ Similar studies have shown that DSPP mRNA was highly expressed in freshly isolated odontoblast/pulp tissue, but was not detect in cultured dental papilla cells derived from rat incisors.^(43,44) It is only when DPSCs are induced, either *in vitro*,⁽⁴⁵⁾ or by *in vivo* transplantation to form an ordered dentin matrix that DSPP is expressed.⁽⁴⁾

15

In vitro studies of *ex vivo* expanded BMSSCs and DPSCs supported the notion that their progeny were morphologically similar to cultured perivascular cells having a bi-polar fibroblastic, stellar or flat morphology, rather than a polygonal endothelial-like appearance. In addition, we have previously shown that the progeny of BMSSC- and DPSC-derived colonies exhibit heterogeneous staining for both CD146 and α -smooth muscle actin, but lack expression of the endothelial markers, CD34 and von Willebrand Factor, *in vitro*.⁽⁴⁾

20

The observations that two different mesenchymal stem cell populations such as BMSSCs and DPSCs harbour in perivascular niches may have further implications for identifying stem cell populations in other adult tissues. Recent findings have identified human "reserve" multipotent mesenchymal stem cells in connective tissues of skeletal muscle, and dermis derived from human fetal and adult samples.⁽⁵⁶⁾ However the exact location, developmental potential and ontogeny of these stem cells is still largely unknown. In the present study, identification of mesenchymal stem cell niches in bone marrow and dentin pulp may help elucidate the

25

fundamental conditions necessary to selectively maintain and expand primitive multi-potential populations *in vitro*, in order to direct their developmental potentials *in vivo*.

EXAMPLE 2

- 5 *Adult human bone marrow MPC are distinct from stromal precursor cells, haematopoietic stem cells and angioblasts by their high expression of the STRO-1 antigen and lack of CD34 expression*

Postnatal bone marrow appears to be a hub of residential stem and precursor cell types responsible for blood cell formation (haematopoietic stem cells), endothelial development
10 (angioblast), and connective tissue/stromal differentiation (stromal precursor cells/bone marrow stromal stem cells/mesenchymal stem cells). Recent work by our group (Gronthos et al. 2003; Shi and Gronthos 2003) has, for the first time, purified and characterised human multipotential bone marrow mesenchymal precursor cells (MPC) based on their high expression of the STRO-1 antigen and by their co-expression of the immunoglobulin
15 superfamily members, VCAM-1 (CD106) and MUC-18 (CD146). Early studies by Simmons and Torok-Storb (1991a and b), have shown that bone marrow-derived STRO-1⁺ stromal precursor cells, with the capacity to form adherent colonies *in vitro*, also expressed the haematopoietic stem cell marker, CD34, albeit at low levels. These studies used CD34 antibody-complement mediated cell lysis to eliminate a high proportion of adherent colony-
20 forming cells in marrow aspirates (Simmons and Torok-Storb 1991b). It is important to note that while the STRO-1 antibody was generated following immunisation of mice with human CD34⁺ bone marrow cells, this may have arisen due to the fact that the STRO-1 antigen is also expressed at moderate to low levels on CD34⁺/Glycophorin-A⁺ nucleated red cells and CD34⁺/CD20⁺ B-lymphocytes. We now offer direct evidence, using sophisticated
25 fluorescence activated cell sorting technology that multipotential adult human bone marrow MPC express high levels of STRO-1, but lack expression to the stromal precursor cell, haematopoietic stem cell and angioblast marker (CD34), the leukocyte antigen (CD45), and the nucleated red cell marker (Glycophorin-A) (Figure 6A-C). These data demonstrate that adult human bone marrow-derived MPC are a novel stem cell population, distinct from more
30 mature stromal precursor cells, haematopoietic stem cells and angioblast (Figure 7).

Unless otherwise indicated the materials and methods of this example are the same as those for Example 1.

- 5 Figure 6. Expression of CD34, CD45 and Glycophorin-A on STRO-1 positive bone marrow mononuclear cells. Representative histograms depicting typical dual-colour flow cytometric analysis profiles of STRO-1 positive bone marrow mononuclear cells isolated initially by magnetic activated sorting and co-stained with antibodies directed against CD34 (A), CD45 (B) or Glycophorin-A (C). The STRO-1 antibody was identified using a goat anti-murine IgM-fluorescein isothiocyanate while CD34, CD45 and Glycophorin-A were identified using a goat anti-murine IgG- phycoerythrin. The high expressing STRO-1 fraction which contained the clonogenic MPC population was isolated by fluorescence activated cell sorting based on regions R1 and R2.
- 10
- 15 Figure 7. Bone marrow MPC are STRO-1 bright, CD34 negative, CD45 negative and Glycophorin-A negative. The graph depicts the results of in vitro adherent colony formation assays performed for each of the different sorted STRO-1 bright populations selected by their co-expression or lack of either the CD34, CD45 or Glycophorin-A antigens, based on regions R1 and R2 as indicated in Figure 6. These data are expressed as the mean incidence of colony-forming units for each cell fraction averaged from two separate experiments.
- 20

EXAMPLE 3. Identification of multipotential MPC in different human tissues

- While the existence and precise location of MPC in different tissues is largely unknown, we have recently demonstrated that MPC appear to reside in a perivascular niche in human bone marrow and dental pulp tissues (Shi and Gronthos 2003). These observations were based on a combination of immunohistochemical and immunoselection methods to identify and isolate different MPC populations based on their expression of the mesenchymal stem cell marker, STRO-1, the smooth muscle and pericyte markers, CD146, alpha-smooth muscle actin and the pericyte specific marker, 3G5. We have now extended these studies demonstrating the co-localization of STRO-1/CD146, STRO-1/alpha-smooth muscle actin,
- 25
- 30

and 3G5/CD146 antigens in a wider variety of tissues including heart, liver, kidney, skin, spleen, pancreas, lymph node (Figure 8).

To confirm our earlier findings that MPC can be derived from non-bone marrow tissue such as dental pulp, we used fluorescence activated cell sorting to isolate different MPC populations from adult human peripheral adipose. Single cell suspensions were obtained following digestion of the adipose tissue with collagenase and dispase as previously described (Shi and Gronthos 2003). The adipose-derived cells were then incubated with antibodies reactive against STRO-1, CD146 and 3G5. Cell populations were then selected by FACS, based on their positivity (region R3) or negativity (region R2) to each marker and then plated into regular growth medium (Shi and Gronthos 2003) to assess the incidence of adherent colony-forming cells in each cell fraction (Figure 9). Following 12 days of culture, colonies (aggregates of 50 cells or more) were scored and displayed as the number of colonies per 10^5 cells plated for each cell fraction. Our data demonstrated that MPC can be derived from adipose tissues based on their expression of STRO-1/3G5/CD146 antigens (Figure 10). Dual colour flow cytometric analysis confirmed that only a minor proportion of adipose-derived cells co-expressed STRO-1/CD146 and 3G5/CD146 (Figure 11). These findings are consistent with our previous observations that MPC can be isolated from both bone marrow and dental pulp tissue based on the same set of perivascular markers (Shi and Gronthos 2003). Furthermore, we provide evidence demonstrating that adipose derived MPC isolated by CD146 selection have the capacity to differentiate into different tissues such as bone, fat and cartilage (Figure 12), as previous described (Gronthos et al. 2003).

Recent findings examining the existence of MPC in unrelated tissues such as skin has also been examined to further strengthen our hypothesis. Single cell suspensions were obtained following digestion of full thickness human skin with collagenase and dispase as described above for human adipose tissue. The skin-derived cells were then incubated with antibodies reactive against STRO-1, CD146 and 3G5 identified using either a goat anti-murine IgM or IgG- phycoerythrin. Cell populations were then selected by FACS, based on their positivity (region R3) or negativity (region R2) to each marker and then plated into regular growth

medium (Shi and Gronthos 2003) to assess the incidence of adherent colony-forming cells in each cell fraction (Figure 13). Following 12 days of culture, colonies (aggregates of 50 cells or more) were scored and displayed as the number of colonies per 10^5 cells plated for each cell fraction. The data demonstrated that MPC can also be derived from skin based on their
5 expression of STRO-1/3G5/CD146 antigens (Figure 10). Collectively these data suggest that multipotential MPC can be identified and isolated in virtually all vascularised tissues derived from postnatal human tissue based on a common phenotype.

Unless otherwise indicated the materials and methods of this example are the same as those
10 for Example 1.

Figure 8. Reactivity of perivascular makers in different human tissues. Dual-colour immunofluorescence staining demonstrating reactivity of (A) STRO-1 and CD146, (B) STRO-1 and alpha-smooth muscle actin, and (C) 3G5 and CD146, on blood vessels and
15 connective tissue present on spleen, pancreas (Panel I), brain, kidney (Panel II), liver, heart (Panel III) and skin (Panel IV) 20X. The STRO-1 and 3G5 antibodies were identified using a goat anti-murine IgM-Texas Red while CD146 and alpha-smooth muscle actin were identified using a goat anti-murine or IgG-fluorescein isothiocyanate. Co-localization is indicated by overlapping areas of yellow and orange fluorescence (white arrows).

20
Figure 9. Isolation of adipose-derived MPC by FACS. Representative flow cytometric histograms depicting the expression of STRO-1, CD146 and 3G5 in fresh preparations of peripheral adipose-derived single-cell suspensions generated following collagenase/dispase digestion as previously described (Shi and Gronthos 2003). The antibodies were identified
25 using either a goat anti-murine IgM or IgG-phycoerythrin. Cell populations were then selected by FACS, based on their positivity (region R3) or negativity (region R2) to each marker and then plated into regular growth medium to assess the incidence of adherent colony-forming cells in each cell fraction.

Figure 10. Clonogenic adipose-derived MPC are positive for STRO-1/3G5/CD146. The bar graph depicts the number of clonogenic colonies retrieved from single cell suspensions of enzymatically digested human peripheral adipose tissue, following fluorescence activated cell sorting, based on their reactivity to antibodies that recognize STRO-1, CD146, and 3G5 (Figure 9), then cultured in standard growth medium as previously described for bone marrow and dental pulp tissue (Shi and Gronthos 2003). The data are expressed as the number of colony-forming units obtained per 10^5 cells plated in the positive and negative cell fractions averaged from two separate experiments.

- 10 Figure 11. Immunophenotypic analysis of adipose-derived MPC. Representative flow cytometric histograms depicting the co-expression of STRO-1 and CD146 (A) and 3G5 and CD146 in fresh preparations of peripheral adipose-derived single-cell suspensions generated following collagenase/dispase digestion. The STRO-1 and 3G5 antibodies were identified using a goat anti-murine IgM-phycoerythrin while CD146 was identified using a goat anti-
15 murine IgG-fluorescein isothiocyanate. Approximately 60% and 50% of the CD146 positive cells co-express STRO-1 and 3G5, respectively. These data suggest that 10% or more of the CD164 positive cells co-express STRO-1 and 3G5.

Figure 12. Developmental potential of purified Adipocyte-derived MPC *in vitro*.

- 20 Preparations of primary MPC cultures derived from STRO-1⁺/CD146⁺ adipose cells were re-cultured either in standard culture conditions (A), osteogenic inductive medium (B), Adipogenic inductive medium (C) or condrogenic conditions (D) as previously described Gronthos et al. 2003. Following two weeks of multi-differentiation induction, the adipocyte-derived MPC demonstrated the capacity to form bone (B; Alizarin positive mineral
25 deposits), fat (C; Oil Red O positive lipid) and cartilage (D: collagen type II matrix).

- Figure 13. Isolation of skin-derived MPC by FACS. Representative flow cytometric histograms depicting the expression of STRO-1, CD146 and 3G5 in fresh preparations of full thickness skin-derived single-cell suspensions generated following collagenase/dispase
30 digestion. The antibodies were identified using either a goat anti-murine IgM or IgG-

phycoerythrin. Cell populations were then selected by FACS, based on their positivity (region R3) or negativity (region R2) to each marker and then plated into regular growth medium to assess the incidence of adherent colony-forming cells in each cell fraction.

- 5 Figure 14. Clonogenic skin-derived MPC are positive for STRO-1^{bri}/3G5/CD146. The bar graph depicts the number of adherent colonies recovered from single cell suspensions of enzymatically digested human skin, following fluorescence activated cell sorting, based on their reactivity to antibodies that recognize STRO-1, CD146, and 3G5, then cultured in standard growth medium as previously described for bone marrow and dental pulp tissue
10 (Shi and Gronthos 2003). The data are expressed as the number of colony-forming units obtained per 10⁵ cells plated in the positive and negative cell fractions averaged from two separate experiments.

EXAMPLE 4. *Immunophenotypic analysis of ex vivo expanded human bone marrow*

15 *mesenchymal precursor cells*

We have previously reported that multipotential mesenchymal precursor cells (MPC) can be purified from adult human bone marrow mononuclear cells based on the phenotype STRO-1^{bright}/VCAM-1 (CD106)⁺ or STRO-1^{bright}/MUC-18 (CD146)⁺ (Gronthos et al. 2003; Shi and Gronthos 2003). The MPC population can be readily propagated in vitro under defined
20 culture conditions (Gronthos et al. 2003). We now present data characterising the ex vivo expanded MPC progeny based on markers associated with different cell lineages, at both the mRNA and protein level, using reverse transcriptase-polymerase chain reaction (RT-PCR) and flow cytometric analysis, respectively.

- 25 In the first series of experiments, semi-quantitative RT-PCR analysis was employed to examine the gene expression profile of various lineage-associated genes present in the cultured MPC populations (Figure 15). Relative gene expression for each cell marker was assessed with reference to the expression of the house-keeping gene, GAPDH, using ImageQuant software (Figure 15 B). In addition, single-colour flow cytometric analysis was
30 used to examine the protein expression profile of ex vivo expanded MPC based on their

expression of cell lineage-associated markers (Figure 15 A). A summary of the general phenotype based on the gene and protein expression of the cultured MPC is presented in Table 1. Direct comparison of the gene expression profile of MPC described in the present patent demonstrated clear differences between this cell population and mesenchymal stem cells (MSC) previously described by Pittenger et al. 1999, (Table 1).

Unless otherwise indicated the materials and methods of this example are the same as those for Example 1.

Figure 15 A. Immunophenotypic expression pattern of ex vivo expanded bone marrow MPC. Single cell suspensions of ex vivo expanded bone marrow MPC were prepared by trypsin/EDTA treatment then incubated with antibodies identifying cell lineage-associated markers. For those antibodies identifying intracellular antigens, cell preparations were fixed with cold 70% ethanol to permeabilize the cellular membrane prior to staining for intracellular markers. Isotype matched control antibodies were treated under identical conditions. Flow cytometric analysis was performed using a COULTER EPICS instrument. The dot plots represent 5,000 listmode events indicating the level of fluorescence intensity for each lineage cell marker (bold line) with reference to the isotype matched negative control antibodies (thin line).

20

Figure 15 B. Gene expression profile of cultured MPC. Single cell suspensions of ex vivo expanded bone marrow MPC were prepared by trypsin/EDTA treatment and total cellular RNA was prepared. Using RNeasy extraction method total RNA was isolated and used as a template for cDNA synthesis, prepared using standard procedure. The expression of various transcripts was assessed by PCR amplification, using a standard protocol as described previously (Gronthos et al. 2003). Primers sets used in this study are shown in Table 2. Following amplification, each reaction mixture was analysed by 1.5% agarose gel electrophoresis, and visualised by ethidium bromide staining. Relative gene expression for each cell marker was assessed with reference to the expression of the house-keeping gene, GAPDH, using ImageQuant software.

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Figure 16. *Ex vivo* expanded STRO-1^{bri} MPC can develop into arterioles *in vitro*. Single cell suspensions of *ex vivo* expanded bone marrow STRO-1^{bri} and STRO-1^{dull} MPC were prepared by trypsin/EDTA treatment then plated into 48-well plates containing 200µl of matrigel. The STRO-1^{dull} (A) and STRO-1^{bri} (B) MPC were plated at 20,000 cells per well in serum-free medium (Gronthos et al. 2003) supplemented with the growth factors PDGF, EGF, VEGF at 10ng/ml. Following 24 hours of culture at 37°C in 5% CO₂, the wells were washed then fixed with 4% paraformaldehyde. Immunohistochemical studies were subsequently performed demonstrated that the cord-like structures expressed alpha-smooth muscle actin identified with a goat-anti-murine IgG horse radish peroxidase antibody.

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Table 1. Comparison between cultured human Mesenchymal Precursor Cells (MCP's) and cultured human Mesenchymal Stem Cells (MSC's) following *ex vivo* expansion. Antigens found to be present on cell surface, intracellular or in the extra cellular matrix. MPCs express markers of tissues with different developmental origin, ie. ECT-ectoderm, MES- mesoderm and END – endoderm.

15

ANTIGEN	MSC	MPC	Differentiated Cell Type.
STRO-1	-ve	+ve	
Collagen II	-ve	+ve	Chondrocyte (MES)
Collagen IV	-ve	+ve	Fibroblast (MES)
Laminin	-ve	+ve	Fibroblast (MES)
Bone Sialoprotein (BSP)	-ve	+ve	Osteoblast (MES)
Osteocalcin (OCN)	-ve	+ve	Osteoblast (MES)
Nestin	ND	+ve	Neural (ECT)
Glial Fibrillary Acidic Protein (GFAP)	ND	+ve	Neural (ECT)
CBFA1	-ve	+ve	Osteoblast (MES)
Osterix (OSX)	ND	+ve	Osteoblast (MES)
Osteocalcin (OCN)	-ve	+ve	Osteoblast (MES)
Sox9	ND	+ve	Chondrocyte (MES)
Collagen X (COL X)	+ve	+ve	Chondrocyte (MES)
Leptin	ND	+ve	Adipose (MES)
GATA-4	ND	+ve	Cardiomyocyte (MES)
Transferrin (TFN)	ND	+ve	Hepatocyte (END)
Flavin Containing Monooxygenase (FCM)	ND	+ve	Hepatocyte (END)

Table 2. RT-PCR primers and conditions for the specific amplification of human mRNA

Target Gene	Sense/ Antisense (5 -3) Primer Sequences	Product Size
GAPDH	CACTGACACGTTGGCAGTGG/ [SEQ ID NO. 7] CATGGAGAAGGCTGGGGCTC [SEQ ID NO. 8]	417
Leptin	ATGCATTGGGAACCCTGTGC/ [SEQ ID NO. 9] GCACCCAGGGCTGAGGTCCA [SEQ ID NO. 10]	492
CBFA-1	GTGGACGAGGCAAGAGTTTCA/ [SEQ ID NO. 11] TGGCAGGTAGGTGTGGTAGTG [SEQ ID NO. 12]	632
OCN	ATGAGAGCCCTCACACTCCTC/ [SEQ ID NO. 13] CGTAGAAGCGCCGATAGGC [SEQ ID NO. 14]	289
GFAP	CTGTTGCCAGAGATGGAGGTT/ [SEQ ID NO. 15] TCATCGCTCAGGAGGTCCTT [SEQ ID NO. 16]	370
Nestin	GGCAGCGTTGGAACAGAGGTTGGA/ [SEQ ID NO. 17] CTCTAAACTGGAGTGGTCAGGGCT [SEQ ID NO. 18]	460
GATA-4	GACTTCTCAGAAGGCAGAG/ [SEQ ID NO. 19] CTATCCTCCAAGTCCCAGAG [SEQ ID NO. 20]	800
PDGFβ-R	AATGTCTCCAGCACCTTCGT/ [SEQ ID NO. 21] AGCGGATGTGGTAAGGCATA [SEQ ID NO. 22]	650
Osterix	GGCACAAAGAAGCCGTACTC/ [SEQ ID NO. 23] CACTGGGCAGACAGTCAGAA [SEQ ID NO. 24]	247
COL X	AGCCAGGGTTGCCAGGACCA/ [SEQ ID NO. 25] TTTTCCCACTCCAGGAGGGC [SEQ ID NO. 26]	387
SOX9	CTC TGC CTG TTT GGA CTT TGT/ [SEQ ID NO. 27] CCT TTG CTT GCC TTT TAC CTC [SEQ ID NO. 28]	598
Ang-1	CCAGTCAGAGGCAGTACATGCTA AGAATTGAGTTA/ [SEQ ID NO. 29] GTTTTCCATGGTTTTGTCCCGCAGTA [SEQ ID NO. 30]	300

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